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RESEARCH ARTICLE

Insulin inhibits the JNK mediated cell death via upregulation of AKT expression in Schwann cells grown in hyperglycemia

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BACKGROUND: Schwann cells (SCs) are the glial cells of the peripheral nervous system, which forms a thick insulating structure around the axons. Hyperglycemia is known physiologic conditions in both type I and type II diabetes which causes diabetic neuropathy. But the SC possesses insulin receptors even though glucose uptake is independent of insulin. Since the insulin level is highly altered in diabetes, it is of greater importance to evaluate their role in the Schwann cell survival and death.
METHODS: Schwann cells were isolated from neonatal pups and grown with and without insulin in hyperglycemic medium to mimic diabetic condition for 24 and 48 h. We studied the cell viability using 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) and mitochondrial membrane potential (MMP) assay at different time interval on SCs. We also studied the protein and gene expression of Protein Kinase B (AKT) and Jun N-terminal kinase (JNK), which are greatly involved in cell survival and cell death respectively.
RESULTS: The result shows that, high glucose levels for 48 h decrease the SC viability. Hyperglycemic condition induces the SC death by increasing the JNK expression which in turn reduces the MMP of glial cells. However, insulin administration for SCs grown in high glucose condition can reduce the JNK expression by activating AKT signaling pathway.
CONCLUSION: These observations demonstrate that the proper insulin balance is required for Schwann cells survival in hyperglycemic condition. Therefore, altered insulin signaling can be one of the reasons for demyelination of peripheral neurons in diabetic neuropathy.

Keywords insulin, schwann cells, apoptosis, JNK, AKT, diabetic peripheral neuropathy

Introduction

Schwann cells (SCs) are the type of glial cells of the peripheral nervous system derived from the neuronal crust, through different intermediate stages. Differentiation of SC plasma membrane forms the myelin sheath around the axons of peripheral neurons (Jessen and Mirsky, 2005). It is well known that a variety of neurotropic factors like NGF, BDNF and neurotrophin-3 (NT-3) are synthesized and released by SCs (Cai et al., 2010; Denarier et al., 2005). Along with myelination, SCs maintain the structural and physiologic integrity of peripheral neurons (Bhatheja and Field, 2006).

High glucose environment is the chronic pathological complication of Diabetic peripheral neuropathy. Hyperglycemia stimulates several cellular pathways like polyol pathway leading high fructose accumulation, increased advanced glycation end products, protein kinase C activation and mitochondrial dysfunction results in increased the cellular stress and more ROS (Vincent, et al., 2004). Earlier studies on DRG cells shows that exposure of dorsal root ganglion (DRG) cells in 20mM glucose concentration medium for 6 h can cause lipid peroxidation and hyperglycemic-induced superoxide generation that inhibit the TCA cycle. Parallel to this mitochondrial membrane depolarization and chromatin condensation leads to the apoptosis of DRG cells (Zhang et al., 2010). Even though DRG cells increase the production of cytosolic antioxidant enzymes in response to hyperglycemia, they are insufficient to prevent the neuronal cell death leading to neuropathy. Apoptosis is an event of cell death, which can be induced by various cell stimuli. Signaling cascades, such as c-Jun N-terminal kinase (JNK) belongs to MAP-kinases involved in regulation of neuronal plasticity, regeneration and cell death (Barr and Ramirez, 2016). Phosphorylation of JNK can activate BAX a known pro-apoptotic protein leads to mitochondrial mediated apoptosis (Dhanasekaran and Reddy,

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2008). However, phosphatidyl-inositol 3-kinase (PI3K)-protein kinase B (AKT) pathway plays a vital role in cell survival by preventing cells from early apoptosis by activating anti apoptotic proteins. These pathways activate downstream kinases consist of complex cellular signaling that determines the fate of neuronal cells (Campana, 1999; Manning and Cantley, 2007).

In the nervous system, insulin has a vital role in the survival and maintenance of neuronal cells, since glucose uptake is insulin independent in Schwann cells. In our early studies we have reported the presence of insulin receptors on Schwann cells and their correlated expression with myelin proteins (Shetter et al., 2011; Shettar and Muttagi, 2012; Rachana et al., 2016) and recently we identified the altered receptor tyrosine kinases expression involved in the failure of insulin signal transduction in diabetic peripheral neuropathy (DPN) (Manu et al., 2017). Therefore, to evaluate the neurotrophic role and importance of insulin, we used in vitro SC culture in high glucose medium to mimic the stress induced for peripheral glial cells in diabetic neuropathy.

Insulin level is greatly altered in diabetes mellitus, but, it is not completely elucidated the role in controlling the cellular mechanisms required for SC proliferation, survival and maintenance. In the present study, we demonstrated the insulin importance in the proliferation of SC and their role in rescuing the cells from hyperglycemic mediated mitochondrial dysfunction. Further, we tested and proved the hypotheses that insulin increase the SC viability by upregulating the AKT pathway and withdrawal of insulin in high glucose condition induces the activation of JNK apoptotic pathways. Taken together, these results suggest the significance of insulin in rescuing the SCs from hyperglycemia induced cell death leading to demyelination of peripheral neurons and these can be a therapeutic target in DPN.

Materials and methods

Materials

All antibodies were obtained from Abcam (UK). Real time PCR reagents were purchased from Invitrogen (USA). PVDF membrane is purchased from Millipore (India). DMEM and FBS were obtained from Gibco (USA). DPBS and skimmed milk powder were procured from Himedia (India). Insulin and all other chemicals were obtained from Sigma (USA) unless mentioned.

Culture of primary rat Schwann cells

SC culture was obtained using a previously described Brockes method (Brockes et al., 1979) with slight modifications (Shettar and Muttagi, 2012). Briefly, Neonatal rat sciatic nerves were dissected under aseptic condition and placed in Dulbecco's modified Eagle's medium (DMEM). Nerves were dissociated in collagenase and trypsin and then plated on poly-L-lysine treated 35 mm plates. Cells were grown in medium containing 90% DMEM, 10% fetal calf serum and kept at 5% CO_2 and 95% air atmosphere at 37°C. After obtaining the confluency, cells was resuspended in serum free 1:1 DMEM and Ham's F12 media. The cell density was ~5000 per well in 96 well plates, 30 000–40 000 cells per 18 mm coverslip pre-coated with poly- l-lysine in a 6-well plate for immunocytochemical studies. For protein and mRNA expression, cells were plated at a density of 10×10^5 cells/ $75cm^2$ tissue culture flask. After attaining 90% confluence the cells were used for experiments. The purity of the SCs was confirmed with S-100 protein staining technique and according to their morphological appearance like phase bright, narrow, bipolar structure under microscopic view.

MTT assay

The SC viability was studied using 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetra- zolium bromide (MTT) assay. Cells were seeded in 96-well plate and cultured at 37°C for 24 h and 48 h under normal glucose (5.6 mM), high glucose condition (40 mM) with and without 10nM insulin. Then, 10 µL of MTT was added and the culture was incubated for another 2 h at 37°C. Subsequently, the medium was discarded and insoluble dark blue formazon was dissolved in DMSO and quantified at 570 nm using a Bio-Rad (USA) microplate reader.

Mitochondrial membrane potential assay

Mitochondrial membrane potential (MMP) was measured at 24 h, 48 h and 72 h using fluorescent potentiometric dye JC-1 as described previously (Rohitkumar et al., 2015). SCs were treated with high glucose and insulin (10 nM) for different time intervals and then washed and labeled for 45 min with 10 µM JC-1 at 37°C. Cells were washed three times, resuspended in PBS, and fluorescence was measured. Subsequently, the changes in fluorescence were monitored at two different wavelengths. The ratio of the reading at 590 nm (red fluorescence of JC-1 aggregates) to 530 nm (green fluorescence of diluted JC-1) (590: 530 nm ratio) was considered as the relative $\Delta\Psi m$ value.

Immunofluorescence

SCs grown for 24 h and 48 h in 40mM of glucose with and without 10nM insulin were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.5% Saponin for 15 min. The cells were blocked with DPBS containing 10% FBS for 1 h at room temperature and incubated overnight with a 1:400 and 1:200 dilution of anti-pJNK and anti-pAKT respectively in DPBS containing 10% FBS. After the primary antibody incubations, 1:50 dilution of

FITC-conjugated goat anti-rabbit was added for 1 h at room temperature. The coverslips were washed in DPBS and mounted on slides using paramount. The fluorescence immunoreactivity was visualized by epifluorescence microscopy (Olympus, Japan) and photographed in the dark. All the experimental images were analyzed in duplicates.

Western Blotting

Western blotting procedures have been described previously (Manu et al., 2017). SCs grown in DMEM and Ham's F12 medium with 40 mM Glucose, in the absence and presence of 10 nM insulin for 24 h and 48 h time interval, were harvested and protein lysate was prepared. Protein were separated by SDS-PAGE gel and electrophoretically transferred to PVDF membrane. After transfer Membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline plus 0.1% Tween-20 (TBS-T) and blotted with anti-pJNK, anti-pAKT and β-actin primary antibody overnight with a dilution of 1:1000, 1:500 and 1:5000 respectively at 4°C. Further blots were washed three times in TBS-T and incubated with secondary antibody conjugated with alkaline phosphatase for 1 h at room temperature, and developed with NBT/BCIP substrate (Genei, India).

RNA isolation and quantitative real time PCR

Following the treatment of SCs in different medium, total RNA was extracted using total RNA isolation kit by strictly following the manufacturer's protocol. RNA quantification was done by measuring the OD at 260 nm using a spectrophotometer (Analytik Jena, Germany). Two step RT PCR was performed. First, Isolated RNA was reverse transcribed to cDNA using pure link RNA mini kit. Next, the expression levels of JNK and AKT were quantified using Syber Green with a StepOne plus real-time PCR system (Applied Biosystems, USA).

2.0 µg of cDNA and power SYBR green master mix (Applied Biosystems, USA) was used to perform real-time PCR amplification. All reactions were run in triplicate. The primer sequences used were as follows:
AKT forward: 5′-TCATTGAGCGCACCTTCCAT-3′
AKT reverse: 5′-TTCTGCAGGACACGGTTCTC-3′
JNK forward: 5′-CTTGCCAGCCTTCGTGTTTC-3′
JNK reverse: 5′-TAGCGGGTACGAGAACTGGA-3′
RPL19 forward: 5′-CGTCCTCCGCTGTGGTAAA-3′
RPL19 reverse: 5′-AGTACCCTTCCTCTTCCCTAT-3′
RPL19 was used as the housekeeping gene for normalization. The relative expressions of genes were analyzed by stepOne™ software v2.2.2. The fold changes were calculated by $2^{(-\Delta\Delta Ct)}$ method.

Statistical analysis

Statistical analyses of the experimental data were carried out by an unpaired student's *t*-test. All the assays were carried out in triplicates. All data were presented as the mean±SEM. Values of $p < 0.05$ were considered significant.

Results

Hyperglycemic condition reduces SC viability

The viability of SC in hyperglycemic condition for 24 h and 48 h was assessed using MTT assay. The SCs were grown in normal, high glucose media (H-G) and high glucose media with insulin (H-G + I). SC grown in H-G medium for 24 h showed 20% decrease cell viability (Fig. 1A) and cells were grown for 48 h showed 45% decreased viability (Fig. 1B) compared to control. Whereas, the SC grown in the H-G + I medium showed 12% and 30% increased viability compared to cells grown in H-G medium for 24 and 48 h respectively. These results suggest that hyperglycemia will induce SC death and insulin administration can increase their viability.

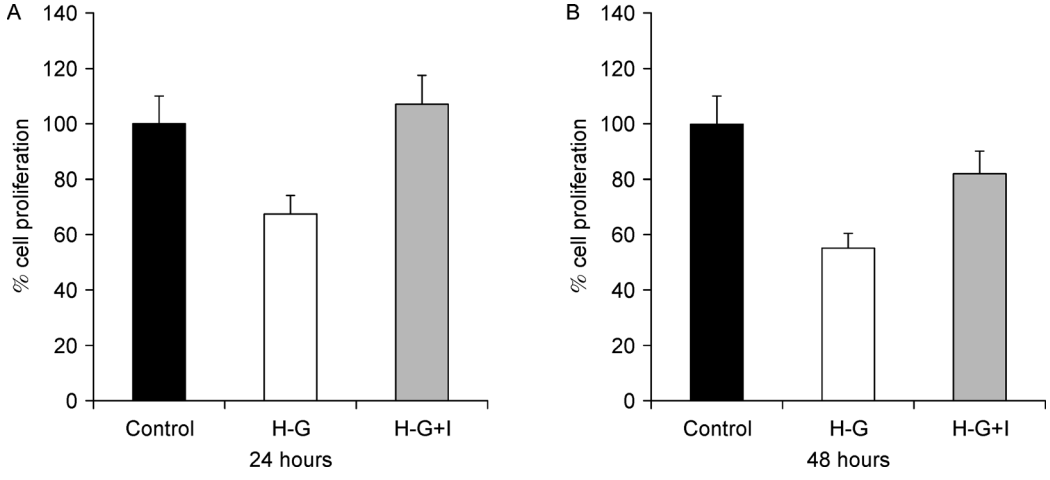

Figure 1 (A and B) Viability of SCs in presence and absence of 10nM of insulin at different glucose concentration for 24 h and 48 h were determined by MTT assay.

Hyperglycemia alters membrane potential and induces JNK mediated cell death

Mitochondrial membrane dysfunction is a known hallmark of apoptosis. We measure the membrane potential of SCs at different condition and time using JC-1 staining. In high glucose condition, about 0.2, 0.3 and 0.5 fold decreased MMP was observed compared to control SC of 24, 48 and 72 h respectively (Fig. 2). Upon the insulin addition, the gradual increased MMP was observed in 48 and 72 h SC compared to cells of high glucose medium. These confirm the reduced MMP in hyperglycemic SCs.

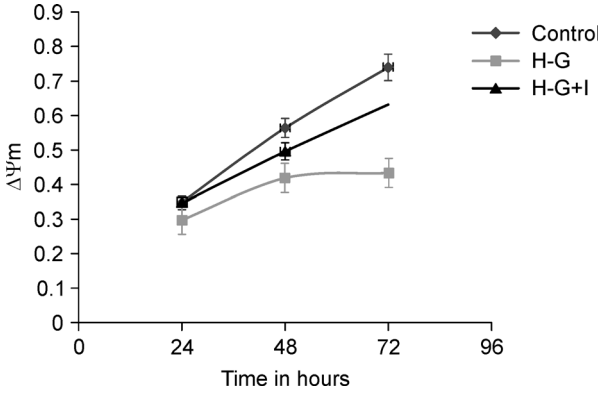

Figure 2 Effect of high glucose in presence and absence of insulin on mitochondrial membrane potential of SCs at different time intervals was measured using 10 μM JC-1 fluorescence dye. All the experiments were done in triplicates.

Further, western blotting and real time PCR techniques were carried to know the JNK protein and gene expression studies. Proteins and total mRNA were isolated from SCs grown in normal, high glucose media (H-G) and high glucose media with insulin (H-G + I). Results showed that SCs grown in hyperglycemic condition for 24 h showed 0.2 fold increased expression of JNK proteins and was increased to 0.9 folds at 48 h compared to control, whereas in the insulin treated cells the expression was decreased to 0.6 and 0.85 folds in 24 h and 48 h compared to high glucose medium (Fig. 3A and B). Along with this total relative mRNA expression of H-G medium for 24 h and 48 h showed the increased expression of 0.6 and 2.0 folds respectively compared to control. SCs grown in the presence of insulin for 24 h and 48 h shows decreased expression of 0.49 and 1.65 folds respectively (Fig. 3C), compared to cells grown in hyperglycemic condition without insulin.

We performed Immunofluorescence with monoclonal antibody to know the SCs JNK expression in hyperglycemic condition. The decreased Schwann cell number and with increased expression of JNK is observed in high glucose medium (Fig. 4C and D). Schwann cells grown in H-G shows more expression of JNK compared to C (Fig. 4A and B), insulin added medium (Fig. 4E and F) showed decreased JNK expression compared to H-G and also increased cell number. These findings indicate the JNK mediated SC death during hyperglycemic condition.

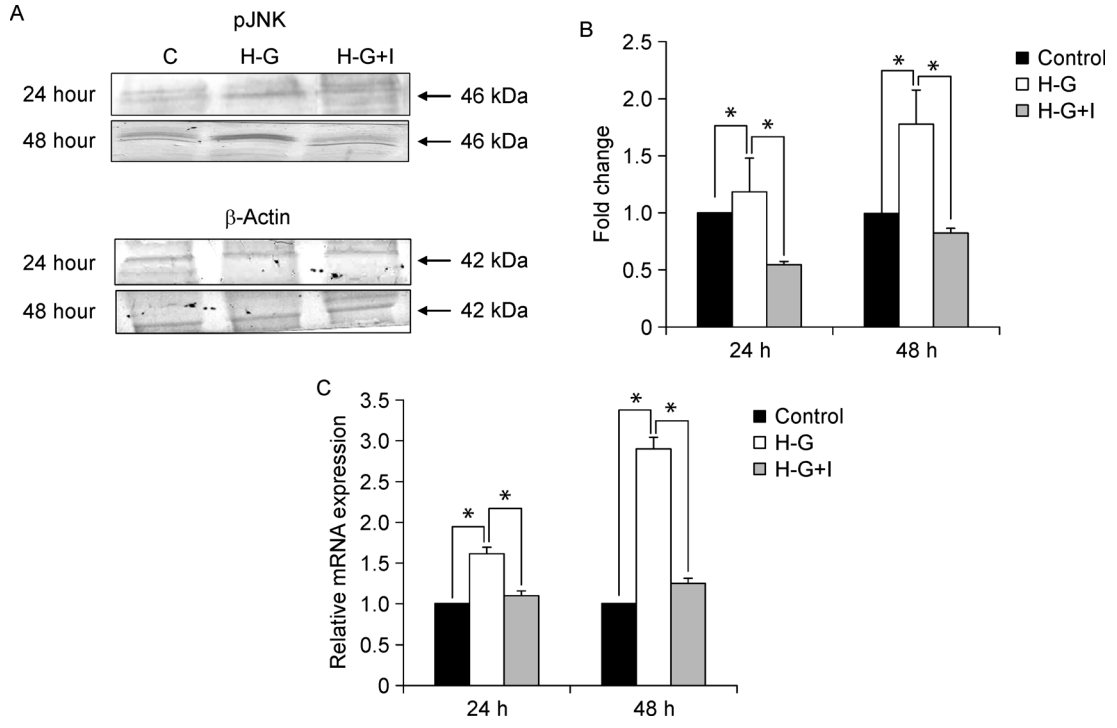

Figure 3 SCs were grown in normal condition (C), high glucose conditions (H-G) and high glucose with 10nM of insulin (H-G + I) for 24 h and 48 h. **(A)** Western blot analysis showing relative fold change in pJNK proteins expression. **(B)** Fold changes were presented as bar chart. **(C)** Real time studies showing relative mRNA expression of JNK. RPL19 is used as internal control. * indicates $p < 0.05$.

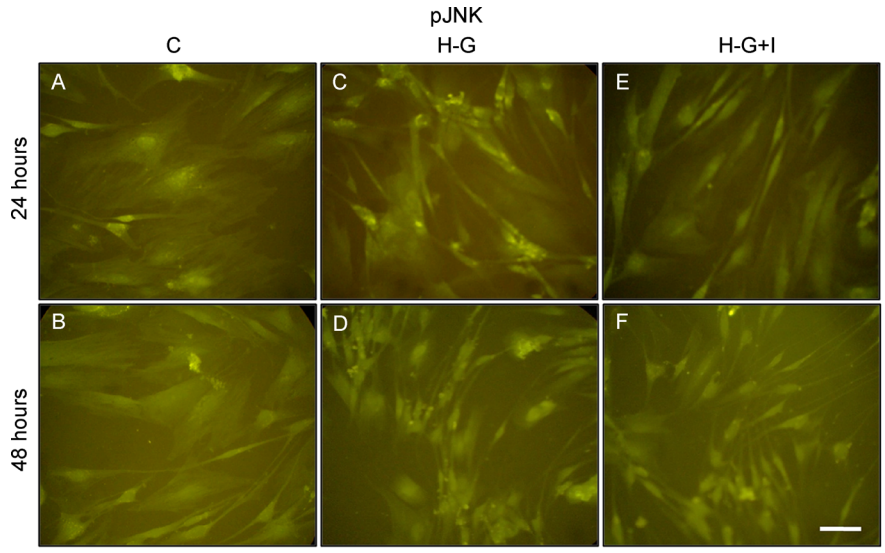

Figure 4 Immunofluorescence expression of pJNK protein in SCs grown for 24 h and 48 h in normal medium [(control) **A and B**], high glucose medium [(40 mM glucose) **C and D**] and insulin added high glucose medium [(40 mM glucose + 10 nM insulin) **E and F**]. Scale bar: 50 μm.

Insulin protects Hyperglycemic Schwann cells by AKT cell survival pathway

AKT signaling pathway is one of the important cell survival pathways, therefore we have measured the expression of AKT in SCs grown in the hyperglycemic medium in the presence and absence of insulin. Western blot was performed on SCs. The AKT expression in H-G cells for 24 h and 48 h showed 0.45 and 0.60 fold decreased expression compared with C and H-G + I showed increases of 0.50 and 0.26 fold compared to H-G cells (Fig. 5A and B). Further AKT mRNA has also showed the significant change, m RNA expression of

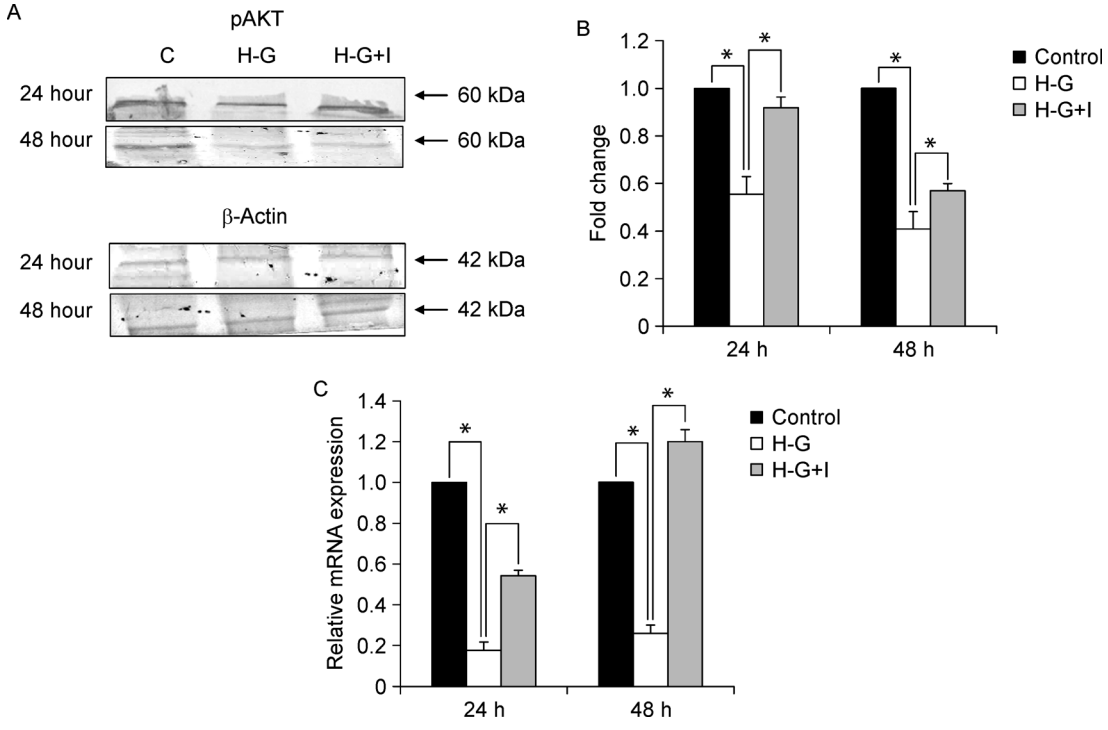

Figure 5 SCs were grown in normal condition (C), high glucose conditions (H-G) and high glucose with 10nM of insulin (H-G + I) for 24 h and 48 h. **(A)** Western blot analysis showing relative fold change in pAKT proteins expression. **(B)** Fold changes were presented as bar chart. **(C)** Real time studies showing relative mRNA expression of AKT. RPL19 is used as internal control. * indicates $p < 0.05$. Scale bar: 50 μm.

H-G cells showed decreased expression of 0.83 and 0.74 fold compared to C, where H-G + I cells of 24h and 48h showed 0.37 and 0.94 fold increased expression compared to H-G SCs respectively (Fig. 5C).

Further, the expression of AKT was determined by immunocytochemistry using FITC conjugated secondary antibody. Compared to control (Fig. 6A and B) the expression of AKT was decreased in SCs of H-G medium (Fig. 6C and D). Where the expression was significantly increased in the SCs of insulin (10nM) added medium (Fig. 6E and F), suggesting that insulin plays a vital role in the AKT signaling pathway which is essential for cell survival.

Discussion

Programmed cell death (PCD) is one of the essential cellular events happens throughout the body to maintain a normal and healthy growth of the tissue. During the myelination event, the SCs that fail to contact the axon will undergo apoptosis, this regulates the glial cell number in maintaining healthy neurons (Nakao et al., 1997). Oxidative stress, mitochondrial membrane depolarization, lipotoxicity and several other aberrant physiologic conditions lead to neuronal damage through PCD in peripheral neurons (Russell et al., 1999). These kind of abnormalities along with impaired metabolic function and protein synthesis dysregulation are considered as main reasons for the pathogenesis of diabetic neuropathy (Kristiansen et al., 2010). The present study shows that the hyperglycemia for the long duration will reduce the SC viability by altering the membrane potential of mitochondria through JNK mediated pathway. Where insulin administration can reverse this mechanism by increasing the expression of AKT, which is a known cell survival pathway.

In previous studies from our laboratory, we reported the correlated expression of myelin proteins with insulin, these findings show the importance of insulin signaling in myelin formation and maintenance (Shettar and Muttagi, 2012; Rachana et al., 2016). Later in the recent report, we have shown, one of the possible reasons behind the failure of insulin signaling in diabetic neuropathy. The increased phosphorylation of IRS2 at serine 731 position and decreased GRB2 adaptor protein activation in response to insulin is resisting the insulin signaling (Manu et al., 2017). In the current study, we performed MTT assay to identify the role of hyperglycemia on SC viability for 24 h and 48 h. The result clearly shows that increased duration of hyperglycemia decreases the SCs viability. Where as in parallel to that, insulin administration for high glucose medium significantly increases the SC viability, indicating that even though glucose uptake is independent of insulin in SCs, it has a vital role in the SC survival, therefore it is preventing the SC death in hyperglycemic condition. Also it is known that hyperglycemic environment can induce the morphological variation among SCs (Delaney et al., 1999). Further, MMP assay showed the decreased membrane potential along with time in the SCs grown in hyperglycemia in the absence of insulin. These findings confirm the altered mitochondrial membrane potential of SCs leading to cell death.

JNK signaling pathway is greatly involved in neuronal apoptosis. NGF withdrawal from the in vitro sympathetic neurons induces the neuronal cell death within 24-48 h by activating JNK pathway, mitochondrial cytochrome C release, and activation of caspase (Kristiansen et al., 2010). In the ischemia-induced neuronal apoptosis, JNK signal regulates the BimL as a downstream substrate for transmis-

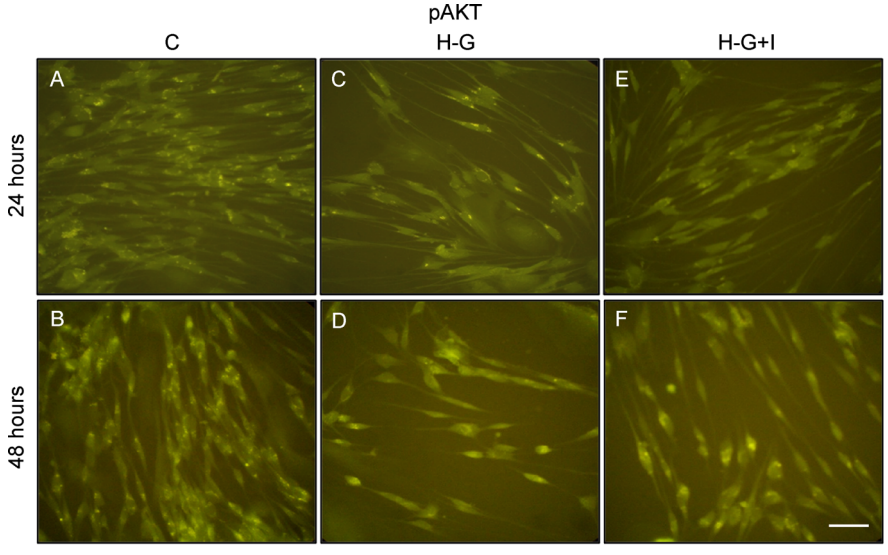

Figure 6 Immunofluorescence expression of pAKT protein in SCs grown for 24 h and 48 h in normal medium [(control) **A and B**], high glucose medium [(40 mM glucose) **C and D**] and insulin added high glucose medium [(40 mM glucose + 10 nM insulin) **E and F**]. Scale bar: 50 μm.

sion of apoptotic signals to Bax and PUMA (Okuno et al., 2004)

Therefore we wanted to know the involvement of JNK signaling pathway in SC death. In hyperglycemic condition, we observed the partial expression of JNK in 24 h and the significant increased expression in 48 h. SCs grown in insulin added high glucose condition exhibited a strong suppression of JNK expression. These results approach us to conclude that SCs will undergo JNK mediated apoptosis in high glucose condition and this can prevail by proper insulin administration.

On the above results and our previous studies on insulin signaling pathway, we intend to know the possible signaling cascade that may involve in SC survival in high glucose condition. In the neuronal cell death induced by oxidative stress, PI3K/AKT pathway plays a protective role by activating C-AMP responsive element binding protein (CREB) and nuclear factor-κB (NF-κB). It can also directly inhibit the apoptotic machinery by phosphorylation at sites of pro apoptotic protein (Morrison et al., 2002; Uranga et al., 2013). In this regard, we carried the experiments to know the frequency of AKT signaling pathway involved in SCs. We clearly observe that decreased expression of AKT activation in high glucose condition is reverted upon the insulin addition. The significant change in AKT gene and protein expression supports the involvement of AKT signaling pathway in resisting the SCs death in hyperglycemic condition.

Our results provide strong evidence that Insulin signal is essential for SC to survival and it also plays a major role in preventing SC death in hyperglycemia. Since JNK and AKT signaling pathway has the opposing effects on neuronal cells, it is important to evaluate their expression in SC. Therefore, these findings indicate that high glucose concentration will induce apoptosis through JNK mediated Map-kinases pathway, where insulin administration increases AKT expression and rescue the SCs from apoptosis. The proper monitoring of insulin concentration in diabetic patients is very much essential to avoid diabetic peripheral neuropathy and also these pathways can be therapeutically targeted to resist the SC death induced by hyperglycemia.

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Compliance with ethics guidelines

Mallahalli S. Manu, Kuruvanthe S. Rachana and Gopal M. Advirao declares that they have no conflict of interest.

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